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(54) Title: VASOACTIVE AMINE BINDING MOLECULES			
(57) Abstract			
According to the present invention, there are provided vasoactive amine binding proteins (VABPs) that specifically bind to vasoactive amines with a dissociation constant of less than 10 ⁻⁷ M and which belong to the same protein family as MS-HBP1, FS-HBP1, FS-HBP2 and D.RET6.			

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VASOACTIVE AMINE BINDING MOLECULES

The present invention relates to vasoactive amine binding molecules (VABMs) and their use in the regulation of the action of vasoactive amines. The invention in particular relates to VABMs which are derived from parasite proteins or derivatives thereof. The present invention also relates to the detection and quantification of vasoactive amines and to the control of diseases and injury caused by parasites in animals and humans, especially those caused by ectoparasites of domestic animals. It further relates to the use of vasoactive binding molecules in the treatment of diseases and allergies. The present invention also relates to the use of recombinant DNA technology to produce VABMs.

15

Vasoactive amines such as histamine and serotonin are mediators of inflammation and regulators of certain physiological processes in animals, including humans. Histamine is present in the secretory granules of mast cells and basophils and is formed by decarboxylation of histidine. It is also present in ergot and plants and may be synthesised synthetically from histidine or citric acid.

The main actions of histamine in humans are stimulation of 25 gastric secretion, contraction of most smooth muscle, cardiac stimulation, vasodilation and increased vascular permeability. In addition to its regulatory role in immune reactions and inflammatory processes, histamine also modulates the production of many cytokines in the body 30 (including those that regulate inflammation) and can interfere with the expression of cytokine receptors. Furthermore, histamine promotes wound healing.

The main pathophysiological roles of histamine are as a 35 stimulant of gastric acid secretion and as a mediator of type I hypersensitivity reactions such as urticaria and hay fever. Histamine or its receptors may also be involved either directly or indirectly in autoimmune disease, e.g.

arthritis, and in tumour growth (Falus, 1994).

Histamine produces its actions by an effect on specific histamine receptors which are of three main types, H₁, H₂ and H₃, distinguished by means of selective antagonist and agonist drugs. Histamine H₁ and H₂ receptor antagonists have clinical uses but at present histamine H₃ receptor antagonists are used mainly as research tools.

- 10 H₁ receptor antagonists (antihistamines) are widely used for treating allergic reactions including allergic rhinitis (hay fever), urticaria, insect bites and drug hypersensitivities. Drugs that lack sedative or muscarinic-receptor antagonist activities are preferred. H₁ receptor antagonists are also
15 used as anti-emetics for the prevention of motion sickness or other causes of nausea including severe morning sickness. Muscarinic-receptor antagonist actions of some antihistamines probably contribute to efficacy but also cause side effects. Some H₁ receptor antagonists are fairly
20 strong sedatives and may be used for this action.

There are numerous undesirable effects of H₁ receptor antagonists. When used for purely antihistamine actions, all the CNS effects are unwanted. When used for their
25 sedative or anti-emetic actions, some of the CNS effects such as dizziness, tinnitus and fatigue are unwanted. Excessive doses can cause excitation and may produce convulsions in children. The peripheral antimuscarinic actions are always undesirable. The commonest of these is
30 dryness of the mouth, but blurred vision, constipation and retention of urine can also occur. Unwanted effects not related to the drugs' pharmacological actions are also seen. Thus gastro-intestinal disturbances are fairly common while allergic dermatitis can follow topical application of these
35 drugs.

H₂ receptor antagonists are frequently used as inhibitors of gastric acid secretion. They are used as the drugs of

choice in the treatment of peptic ulcer, as second line drugs in the treatment of Zollinger-Ellison syndrome and for treating reflux oesophagitis. Unwanted effects have been reported that include diarrhoea, dizziness, muscle 5 pains, transient rashes and hyper-gastrinaemia. Some H₂ receptor antagonists can cause gynaecomastia in men and confusion in the elderly.

Besides these unwanted side effects, some histamine 10 antagonists are troublesome if taken with alcohol or with drugs. For example, the antihistamine Seldane used in combination with antibiotics and antifungals may cause life-threatening side effects.

15 Drugs used to control the actions of histamine are not always effective. The reasons why they may have limited efficacy may relate to the specificity of these drugs for only a subclass of histamine-receptors, particularly when certain conditions require interference with a larger 20 spectrum of receptors. Histamine binding molecules (HBM_s) would compete for histamine binding with all receptors and may thus be more suited for treating certain conditions.

The hormone serotonin (also known as 5-hydroxytryptamine) is 25 both a vasoconstrictor and a neurotransmitter. It can also increase vascular permeability, induce dilation of capillaries and cause the contraction of nonvascular smooth muscle. Serotonin is present in the brain and intestinal tissues and is produced by the pineal gland and by blood 30 platelets. Pathological aspects related to serotonin include abnormal blood pressure, migraine, psychological disorders, respiratory disease and coronary heart disease. Serotonin agonists and antagonists are used to treat some of these disorders, but again often have undesirable side- 35 effects.

There is thus a great need for effective antagonists of vasoactive amines that do not generate the side-effects that

detract from their applicability to the treatment of human and animal disorders.

- There is also a need for the quantification of histamine in,
5 for example, food products, various body fluids (e.g. plasma or urine) or cell culture supernatants to monitor the effects of certain allergens, for example, or to point to a specific antagonistic therapy for an allergic reaction. Currently used systems (radioimmunoassays and ELISAs)
10 utilize antibodies against histamine or against histamine-derivatives. However, histamine is not very immunogenic, making it hard to raise high-affinity antibodies against it, and most of the quantitation systems used today are not very sensitive or require modification of the histamine to be
15 measured (for example by acylation or methylation). The use of HBM^s to replace antibodies in assays like these would provide a highly sensitive system to measure unmodified histamine.
20 Another advantage of HBM^s over anti-histamine antibodies is that they can be used as research tools for the removal of free (unbound) histamine from, for example, cell cultures when studying certain biological processes. Due to the presence of antibody receptors on most cells, antibodies
25 might interfere with the normal functioning of these cells.

It is known that blood-feeding ectoparasites, such as ticks, produce numerous bioactive proteins that immunomodulate the host response to parasite feeding and thereby promote
30 parasite blood-feeding. Such immunomodulatory proteins include immunoglobulin-binding proteins (IGBPs) that are produced in the haemolymph and saliva of ticks and bind to vertebrate host immunoglobulins (Wang and Nuttall, 1995). They also include salivary nitric oxide-carrying haeme
35 protein (nitrophorin) of the triatome bug *Rhodnius prolixus*, which, in addition to carrying nitric oxide, can also bind histamine (Ribeiro and Walker, 1994). Immunomodulatory proteins are also produced by other blood-feeding parasites,

such as mosquitoes and leeches, and venom-producing animals such as snakes and spiders.

We have found that blood-feeding ectoparasites, for example
5 ticks, produce proteins capable of binding to vasoactive amines, particularly histamine and serotonin. These proteins are hereafter referred to as vasoactive amine binding proteins (VABPs).

10 We have isolated from ticks four VABPs which are named herein as MS-HBP1, FS-HBP1, FS-HBP2 and D.RET6 and which are closely related to each other. These proteins are entirely novel and show no significant similarity to any previously described protein. The DNA sequences encoding these
15 proteins or fragments thereof can be used to isolate other related proteins in the same family from the same or different species.

The present invention provides a vasoactive amine binding
20 protein (VABP) that specifically binds to vasoactive amines with a dissociation constant of less than 10^{-7} M and which belongs to the same protein family as MS-HBP1, FS-HBP1, FS-HBP2 and D.RET6.

25 A protein is considered to belong to this family if 40% or more of the amino acids that are completely conserved as identical residues in the alignment of the four VABPs alone, are still completely conserved as identical residues if the protein is included in the alignment, the alignments being
30 obtained using GCG's pileup command (Program manual for the Wisconsin package, 1994; gap creating penalty = 2.50; gap extension penalty = 0.05). Also included as a member of the VABP family are those proteins from haematophagous arthropods that bind histamine with an affinity
35 characterised by a dissociation constant less than 10^{-7} M and contain the sequence motifs D/E A W K/R and Y/C E/D L/I W.

The VABPs of the present invention include natural

biological variants (e.g. allelic variants or geographical variations within the species from which the VABPs are derived).

- 5 The present invention also includes functionally-equivalent derivatives and fragments of the vasoactive amine binding proteins, or of proteins belonging to the same protein family as the VABPs. The VABPs, derivatives and fragments of the present invention are hereafter referred to as
10 vasoactive amine binding molecules (VABMs).

The VABPs according to the invention are strong and specific vasoactive amine binders with dissociation constant values considerably lower than $10^{-7}M$. Previously described high
15 affinity histamine or serotonin receptors are proteins that form part of the membranes of the cells that are targeted by histamine or serotonin (Falus 1994). They thus differ from the non-membrane bound proteins of the present invention.

- 20 The VABPs of the present invention are unrelated to the known immunomodulatory proteins discussed above and have a different method of action. The VABPs of the present invention are secreted from a foreign organism into a mammalian host animal and function as regulators of the
25 host's inflammatory and immune responses. In the case of blood-feeding ectoparasites, such inflammatory and immune responses would otherwise inhibit parasite blood-feeding. This function derives from the ability of the VABPs to bind specifically to vasoactive amines.
30

The VABPs of the present invention may be derived from blood-feeding parasites and venom-producing animals such as venomous snakes and spiders. Preferably, the VABPs of the present invention are derived from blood-feeding
35 ectoparasites. Most preferably, they are derived from ticks.

As stated above, the invention also includes functionally

equivalent derivatives and fragments of the VABPs. 'Functionally equivalent' is used herein to indicate that the derivatives and fragments retain the capacity to bind vasoactive amines or that they contain epitopes which can 5 be used in the development of vaccines that target members of the VABP protein family as defined above. The derivatives and fragments may be derived from native VABPs by single or multiple amino-acid substitution(s), addition(s), insertion(s) and/or deletion(s) or by chemical modification 10 of one or more of the amino-acids, for instance by deglycosylation of glycosylated forms.

For instance, a derivative may include an additional protein 15 or polypeptide fused to the VABP at its amino- or carboxy- terminus or added internally to the VABP. The purpose of the additional polypeptide may be to aid detection, expression, separation or purification of the VABMs or may be to lend additional properties to the VABPs as desired. Examples of potential fusion partners include 20 β -galactosidase, glutathione-S-transferase, luciferase, polyhistidine tags, T7 polymerase fragments and secretion signal peptides.

The VABMs of the present invention can be prepared using 25 known techniques of molecular biology and protein chemistry. For example, the VABMs may be prepared by chemical peptide synthesis. This technique is especially useful for the generation of short peptides derived from the VABPs for use as immunogens. The VABMs may also be prepared using the 30 known techniques of genetic engineering such as site-directed or random mutagenesis as described, for example, by Sambrook et al., 1989. The VABMs may also be synthetically prepared, using organic chemistry techniques, resulting in molecules structurally and functionally 35 mimicking the histamine-binding site of any of the members of the VABP family.

VABMs of the present invention may be prepared in

- recombinant form by expression in a host cell. Such expression methods are well known to those of skill in the art and many are described in detail by Sambrook et al., 1989. A suitable expression vector can be chosen for the 5 host of choice. The vector may contain a recombinant DNA molecule encoding a VABM operatively linked to an expression control sequence that is recognised by the host transcription machinery.
- 10 Suitable hosts include commonly used prokaryotic species, such as *E. coli*, or eukaryotic yeasts that can be made to express high levels of recombinant proteins and that can easily be grown in large quantities. Cell lines grown *in vitro* are also suitable, particularly when using virus- 15 driven expression systems such as the Baculovirus expression system which involves the use of insect cells as hosts. VABMs may also be expressed *in vivo*, for example in insect larvae or in mammalian tissues.
- 20 According to a yet further aspect, the present invention provides for use of such VABMs to bind histamine in mammals, thereby to regulate its action and to control its pathological effects.
- 25 The present invention also includes the use of the VABMs of the present invention as anti-inflammatory agents. Preferably, the VABMs are provided as a pharmaceutical composition including an inert carrier or carriers. The VABM may constitute the sole active component of the composition or can form part of a therapeutic package, such as a component of creams for topical administration to insect, snake or scorpion bites, or to skin affected by dermatitis. The proteins may also be used as carrier

molecules for histamine and histamine-related compounds, in creams, oils, powders or pills, to provide slow release of the bound components.

- 5 The present invention also comprises the use of the VABMs of the present invention for the quantification of histamine levels (for example, in blood, nasal lavage fluid, tissues or food products). The VABMs may be supplied as part of a kit together with means of detection that allow the accurate
- 10 quantification of the histamine in the sample to be tested (for example radiolabelled histamine, anti-VABM-antibodies or enzymes such as alkaline phosphatases, peroxidases or luciferases). Such kits will resemble radioimmunoassay, scintillation proximity assay, or ELISA kits, with the VABMs
- 15 as binding molecules in place of antibodies directed against histamine or against histamine analogues. One aspect of the present invention comprises such kits incorporating the VABMs of the present invention.
- 20 The VABMs of the present invention can also be used for the detection of vasoactive amines. Any technique common to the art may be used in such a detection method and may comprise the use of blotting techniques (Towbin et al, 1979), gel retardation or affinity chromatography. The entire VABP may
- 25 be used, or simply an active binding fragment in order to detect substrate. In another embodiment, the VABP may be fused either genetically or synthetically to another protein such as alkaline phosphatase, luciferase or peroxidase in order to facilitate detection.
- 30 The invention also comprises the use of the VABMs of the present invention as histamine-binding entities bound to a support that can be used to remove, isolate or extract histamine (from body tissues, blood or food products). The
- 35 support may comprise any suitably inert material such as gels, magnetic and other beads, microspheres, binding columns and resins.

The present invention also includes the use of VABMs as tools in the study of inflammation, inflammation-related processes or other physiological effects of vasoactive amines such as the role of histamine in the formation of 5 gastric ulcers or its role in immune reactions. For example, the VABMs may be used for histamine or serotonin depletion in cell cultures or in inflamed animal tissues in order to study the importance of histamine or serotonin in these systems.

10

Metazoan parasites, particularly arthropods and helminths, are also sources of infectious diseases and other injurious effects that have major impacts in human and veterinary medicine. Control of arthropod and helminth parasites 15 currently relies primarily on the use of chemicals such as acaricides and antihelmintics. Attempts have been made to use immunological means of control through the use of vaccine technology. There has been some success in identifying certain protective antigens as potential vaccine 20 candidates, but only a few have as yet come to commercial fruition, most notably for the cattle lungworm *Dictyocaulus viviparous* and the cattle tick *Boophilus microplus*. Despite these developments, there is nonetheless a continuing need for metazoan parasite vaccines and in particular for a 25 vaccine which may be used across a broad range of arthropod and/or helminth genera.

The present invention therefore also provides for the use of the VABMs as defined above as immunogens for use as metazoan 30 parasite vaccines and in particular as protective immunogens in the control of diseases caused by arthropod and other metazoan parasites. Suitable candidates for vaccination include domesticated animals such as cattle, goats, sheep, dogs, cats and other animals which require protection 35 against metazoan parasites, especially ticks. The vaccine may include adjuvants of the type which are well known in the art.

Nucleic acid molecules comprising a nucleotide sequence encoding a VABM of the invention form further aspects of the invention. These molecules include DNA, cDNA and RNA, as well as synthetic nucleic acid species.

5

The cDNAs encoding the four particular VABPs are disclosed herein by way of example and their amino acid sequences are shown in Figures 1 to 4 (nucleotides and amino acids are given in their standard one letter abbreviations).

10

The preferred nucleic acid molecule, according to the invention, comprises a nucleotide sequence identical to or complementary to any one of, or any VABM-encoding portion of, any one of the nucleotide sequences shown in Figures 1 to 4 and 6, or a sequence which is degenerate or substantially homologous therewith, or which hybridises with the said sequence. By 'substantially homologous' is meant sequences displaying at least 60% sequence homology. The nucleic acid sequences according to the invention may be single- or double-stranded DNA, cDNA or RNA. Preferably, the nucleic acid sequences comprise DNA.

'Hybridising sequences' included within the scope of the invention are those binding under non-stringent conditions (6 X SSC/50% formamide at room temperature) and washed under conditions of low stringency (2 x SSC, room temperature, or 2 x SSC, 42°C) or conditions of higher stringency, e.g. 2 x SSC, 65°C (where SSC = 0.15M NaCl, 0.015M sodium citrate, pH 7.2).

30

The invention also includes cloning and expression vectors containing the DNA sequences of the invention. Such expression vectors will incorporate the appropriate transcriptional and translational control sequences, for example enhancer elements, promoter-operator regions, termination stop sequences, mRNA stability sequences, start and stop codons or ribosomal binding sites, linked in frame with the nucleic acid molecules of the invention.

Additionally, it may be convenient to cause the recombinant protein to be secreted from certain hosts. Accordingly, further components of such vectors may include nucleic acid 5 sequences encoding secretion signalling and processing sequences.

Vectors according to the invention include plasmids and viruses (including both bacteriophage and eukaryotic 10 viruses). Many such vectors and expression systems are well known and documented in the art. Particularly suitable viral vectors include baculovirus-, adenovirus- and vaccinia virus-based vectors.

15 A variety of techniques are known and may be used to introduce the vectors according to the present invention into prokaryotic or eukaryotic cells. Suitable transformation or transfection techniques are well described in the literature (Sambrook et al., 1989). In eukaryotic 20 cells, expression systems may either be transient (e.g. episomal) or permanent (chromosomal integration) according to the needs of the system.

Nucleic acid molecules according to the present invention 25 may also be used to create transgenic animals. This may be done locally by modification of somatic cells or by germ line therapy to incorporate heritable modifications.

The invention therefore also includes transformed or 30 transfected prokaryotic or eukaryotic host cells or transgenic organisms containing a nucleic acid molecule according to the invention as defined above.

A further aspect of the invention provides a method for 35 preparing a VABM of the invention which comprises culturing a host cell containing a nucleic acid molecule according to the invention under conditions whereby said protein is expressed and recovering said protein thus produced.

All documents mentioned in the text are incorporated herein by reference.

- 5 Various aspects and embodiments of the present invention will now be described in more detail by way of example, with particular reference to VABPs isolated from ticks, and especially the tick *Rhipicephalus appendiculatus*. It will be appreciated that modification of detail may be made
10 without departing from the scope of the invention.

Brief description of the Figures

Figure 1 is the sequence of FS-HBP1, showing sequencing
15 primers and the sequencing strategy used.

Figure 2 is the sequence of FS-HBP2, showing sequencing primers and the sequencing strategy used.

20 Figure 3 is the sequence of MS-HBP1, showing sequencing primers and the sequencing strategy used.

Figure 4 is the sequence of D.RET6, showing sequencing primers and the sequencing strategy used.

25 Figure 5 is a Coomassie-stained 12% SDS-PAGE gel showing salivary gland extracts from ticks that have been purified on a histamine-binding column. Salivary gland extract of female ticks before (lane A) and after purification (lane B; 30 1 = FS-HBP1, 2 = FS-HBP2); salivary gland extract of male ticks before (lane C) and after purification (lane D; 3 = MS-HBP1). Molecular weight markers are indicated.

Figure 6 shows an alignment of the four cDNA-inferred amino acid sequences of the VABPs, created using the pileup and prettyplot commands of the GCG Wisconsin package.

Figure 7 is a Coomassie-stained 12% SDS-PAGE gel showing

recombinantly-produced VABPs. Lane A, rMS-HBP1; lane B, rFS-HBP2; lane C, rFS-HBP1. Molecular weight markers, from top to bottom, indicate 66, 48.5, 29, 18.4 and 14.2kDa.

- 5 Figure 8 is a western blot of salivary gland extracts taken from female and male ticks.

Figure 9 shows saturation curves and Scatchard plots illustrating the histamine-binding properties of purified
10 VABPs.

Figure 10 is a graph depicting contraction-inhibition experiments performed on guinea pig ileum. Abbreviations used: H = histamine (1.25nmol); wash = Krebs solution.

- 15 About 2nmol of FS-HBP2 was added; about 4nmol (monomer amount) of MS-HBP1 was used.

EXAMPLES

20 Ticks

Ticks were reared according to Jones et al., (1988). All three developmental stages of *Rhipicephalus appendiculatus* and *Dermacentor reticularis* and were fed on Dunkin Hartley guinea pigs except adult *Dermacentor reticularis* which were
25 fed on rabbits. When not feeding, all ticks were maintained at 21°C and 85-90% relative humidity.

Example 1: Identification of proteins

30 Salivary glands were excised from female adult *R. appendiculatus* specimens that had been feeding on guinea pigs for three days. Male ticks were fed for four days. Glands were homogenised in phosphate-buffered saline (PBS; pH7.4), cellular debris was removed by centrifugation for 3 minutes at 10,000g and the supernatant was then applied to
35 a column containing 400ml histamine-agarose suspension (Sigma). Unbound protein was washed out of the column with 10ml PBS containing 5% glycerol and bound protein could then be eluted using 100mM histamine in PBS (2ml). The eluans

were concentrated using a centricon 3 ultrafiltration unit (Amicon).

The extracts were run on a 12% SDS-PAGE gel, identifying
5 two major proteins from female ticks and one from male ticks
(see Figure 5). These proteins were termed female-specific histamine binding proteins 1 and 2 (FS-HBP1 and FS-HBP2) and
male-specific histamine binding protein 1 (MS-HBP1). MS-HBP1 was never detected in female tissues, but was clearly
10 present in the salivary glands of males and nymphs and in whole body homogenates of larvae.

Example 2: Cloning of genes

15 1) cDNA library construction

In order to clone the cDNAs encoding the three proteins of example 1, a cDNA library was constructed. Salivary glands were excised from 20 male and 20 female adult *R. appendiculatus* specimens that had been feeding on guinea pigs for two days. The glands were collected in an Eppendorf tube in dry ice. Messenger RNA was isolated using the FastTrack mRNA isolation kit (Invitrogen).

For synthesis of cDNA and its unidirectional insertion into
25 the Lambda Zap II vector, the Zap cDNA synthesis kit (Stratagene) was used. Prior to insertion into the lambda vector, the cDNA was fractionated over a Sephadryl S-400 (Pharmacia) column. A DNA library (termed d2-I) was constructed using low molecular weight cDNAs (ranging from
30 approximately 100 to 2,000 base pairs). The higher molecular weight fraction was used to construct a second library (d2-II). Packaging utilised Packagene (Promega) packaging extracts in accordance with the manufacturer's instructions. Approximately 1.5×10^6 plaque-forming units
35 (PFU) of each library were amplified in XL-1 Blue cells (Stratagene).

A *Dermacentor reticularis* library was constructed with

salivary gland mRNA from adult females that had fed on rabbits for 3 days. Isolation of mRNA, and cDNA library construction was as described above for the d2-II library, except that the Zap Express (predigested vector) Cloning kit 5 (Stratagene) was used instead of the Lambda Zap II kit.

2a) Screening of the d2-II cDNA library

Phagemids were excised *in vivo* from a fraction of the library and used to generate double-stranded pBluescript 10 SK(-) plasmids in XL1-Blue cells (Stratagene), as described by Short et al., (1988). Colonies were plated out on ampicillin-containing LB agar plates supplemented with 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal, Melford Laboratories, UK) and isopropyl- β -D-thiogalactopyranoside (IPTG, Novabiochem) for blue/white colony selection. About 75 plasmids from white colonies were selected for sequencing. The size of the DNA inserts ranged from 250 to 1000 base pairs as determined by digestion with *Pvu*II and electrophoresis over a 1% agarose gel.

20 Clones FS-HBP1, FS-HBP2 and MS-HBP1 were obtained and partially sequenced. The d2-II library was then screened for additional clones by DNA hybridisation of plaque lifts (Sambrook et al., 1989) with digoxigenin-labelled probes 25 (Boehringer Mannheim). The probes were constructed by random primer labelling using the purified insert from the original clones and detected using anti-digoxigenin antiserum conjugated with alkaline-phosphatase (Boehringer Mannheim). For each original clone, 3 additional clones 30 were isolated and sequenced.

2b) Screening of the *Dermacenter reticularis* cDNA library

Firstly, a DNA probe was constructed. A fraction of the *Dermacenter* library was not inserted into the Zap Express 35 vector, but instead was submitted to PCR, using the degenerate primers 5'- AAYGGNGARCAYCARGAYGCNTGGAA (forward) and 5'- KTRTMRTCNRYCCANARYTCRTA (reverse). These primers were based on conserved domains in the FS-HBP1, FS-HBP2 and

MS-HBP1 cDNAs and proteins.

The PCR consisted of 35 cycles with a 30-second melting step at 95°C, a 30-second primer-annealing step at 50°C and a 30-second extension step at 72°C (Taq polymerase was from Perkin Elmer, deoxynucleotides were from Pharmacia). A single DNA band of the expected size (around 400 base pairs) was obtained, which was labelled with digoxigenin to screen the library (as above). D.RET6 was one of several positive clones.

3) Sequencing

The entire coding and non-coding strands of the FS-HBP1, FS-HBP2, MS-HBP1 and D.RET6 inserts were sequenced. Plasmids were purified from overnight cultures according to Good and Feinstein (1992), alkali-denatured (Mierendorf and Pfeffer, 1987) and sequenced by means of the Sanger dideoxy-mediated chain termination reaction (Sanger and Coulson, 1975). The sequencing strategies are shown in Figures 1-4.

20

3a) FS-HBP1

As shown in Figure 1, the original clone was sequenced from the T3 (forward) and T7 (reverse) primer sites flanking the pBluescript SK(-) polylinker region. Additionally, subclones XVIIIA (comprising nucleotides 221 to 770 of the original insert) and XVIIIB (nucleotides 509 to 770) were sequenced from the T3 site (reactions indicated by T3a and T3b in the figure). Subclones XVIIIC (1 to 221) and XVIIID (1 to 509) were sequenced from the T7 site (T7c and T7d).

30

XVIIIA was created by digestion of the original clone with *Pst*I (cuts at position 221 of the insert and in the upstream polylinker region) followed by religation; XVIIIB by digestion with *Xba*I (cuts at position 509 and upstream of the insert). XVIIIC and XVIIID were obtained using *Eco*RI (cuts upstream of the insert) together with *Pst*I and *Xba*I, respectively and ligating the excised pieces back into pBluescript (SK-) plasmid. The signal sequence is given in

bold lettertype in the figure and the signal cleavage site is indicated by the vertical arrow (↑). The underlined sequence was also obtained by amino terminal sequencing of the expressed protein.

5

3b) FS-HBP2

Figure 2 shows the cDNA and inferred amino acid sequence of the clone FS-HBP2. The original clone was sequenced from the T3 (forward) and T7 (reverse) primer sites, as were 2 10 subclones (52a and 52b) obtained by digestion of 52-1 with *Hinc*II (cuts at position 254, the reactions are indicated by T3b and T7a). *Hinc*II was used in combination with *Xba*I (cuts the polylinker downstream of the insert) for construction of 15 52a (comprising nucleotides 1 to 254) and in combination with *Sma*I (cuts upstream) for construction of 52b (nucleotides 254 to 793). Digestion was followed by blunt-ending with T4 polymerase (New England Biolabs) and religation of the plasmids. Finally, we used forward (-) and reverse (-), insert-specific primers that were identical or 20 complementary to the underlined sequences in the figure.

The polyA tail is in italic, the putative polyadenylation signal is doubly underlined. The signal sequence is given in bold lettertype, the signal cleavage is indicated by the 25 vertical arrow (↑). The underlined sequence was also obtained by amino terminal sequencing of the expressed protein.

3c) MS-HBP1

30 Figure 3 shows the cDNA and inferred amino acid sequence of clone MS-HBP1. The clone was sequenced from the T3 (forward) and T7 (reverse) primer sites flanking the pBluescript SK(-) polylinker region. Additionally, we used forward (-) and reverse (-), insert-specific that were identical or 35 complementary to the underlined sequences in the figure.

The triple line indicates the putative N-glycosylation site. Italics denote the polyA tail and the double line marks the

putative polyadenylation signal. The signal sequence is given in bold lettertype, the signal cleavage is indicated by the vertical arrow (↑). The underlined sequence was also obtained by amino terminal sequencing of the expressed 5 protein.

3d) D.RET6

The cDNA and inferred amino acid sequence of clone D.RET6 is given in Figure 4. The DNA insert was sequenced from the T3 10 (forward) and T7 (reverse) primer sites flanking the pBK-CMV polylinker region and from the forward (-) and reverse (-), insert-specific primers that were respectively identical or complementary to the underlined sequences in the figure. The putative signal sequence is given in bold 15 lettertype, the signal cleavage is indicated by the vertical arrow (↑).

3e) Sequence analysis

Sequence data were analyzed using the GCG sequence analysis 20 software (Program Manual for the Wisconsin Package, 1994). Protein database searches were performed at the National Centre for Biotechnology Information (NCBI) using the BLAST network service.

An alignment of the cDNA-inferred amino acid sequences of 25 the VABPs is shown in Figure 6. This was created using the pileup and prettyplot commands of the GCG software. The mature proteins begin at the underlined amino acids, as determined by N-terminal sequencing of the secreted VABPs 30 (see below), suggesting that the preceding regions represent signal sequences. The calculated molecular weights, excluding signal sequences are 19 442 for FS-HBP1, 19 471 for FS-HBP2, 21 026 for MS-HBP1 and 21 025 for D.RET6. Calculated isoelectric points are 4.0, 3.9, 5.0 and 4.6 35 respectively.

MS-HBP1 has 40% identity (57% similarity) with FS-HBP1, 43% (62%) with FS-HBP2 and 32% (50%) with D.RET6. FS-HBP1 has

66% identity (78% similarity) with FS-HBP2 and 32% (49%) with D.RET6. FS-HBP2 has 39% identity and 56% similarity with D.RET6. These percentages were obtained with the Bestfit command of the GCG software, using gap weight of 3
5 and length weight of 0.1).

The predicted secondary structures are similar for the four proteins, with α -helices prevailing in the amino terminal half of the molecules and relatively more β -sheet and turns
10 in the carboxy terminal half. The lower affinity of FS-HBP1 for (positively-charged) histamine suggests that residues at these positions may form part of the binding site.

Example 3: Recombinant protein expression

15

1) Construction of clones

FS-HBP1, FS-HBP2 and MS-HBP1 were expressed as histidine-tagged proteins (rFS-HBP1, rFS-HBP2 and rMS-HBP1) in *Spodoptera frugiperda* ovarian cells (Sf21).

20

In order to append the His₆ tag, the coding region of FS-HBP1 was first amplified using the polymerase chain reaction (PCR). The PCR consisted of 20 cycles with a 30-second melting step at 95°C, a 30-second primer-annealing step at
25 50°C and a 30 second extension step at 72°C. The forward primer used was:

5'-GCAGGAGCTCGGCACGAG;

the reverse primer was:

5'--TTTACTAGTGATGGTATGATGGATCCCTTCTGGGAGGCAATCACTT.

30 The primers were designed so that a SacI site was added upstream of the start codon, whilst the stop codon was replaced by a BamHI site, followed by 6 histidine codons and an SpeI site comprising a TAG stop codon. The PCR product was cut with SacI and SpeI. The latter enzyme
35 creates a compatible overhang with XbaI, enabling the fragment to be ligated between the SacI and XbaI sites of the pACC129.1 transfer vector (Livingstone and Jones, 1989), generating the plasmid pACC129.1-FS1.HIS. This plasmid

therefore contained the sequence Gly-Ile-(His)₆ appended to the carboxy terminus of the FS-HBP1 translation product.

This plasmid pACC129.1-FS1.HIS was also used for expression 5 of histidine-tagged FS-HBP2 and MS-HBP1. The FS-HBP1 cDNA was deleted using *SacI* and *BamHI* thus leaving the histidine codons intact. An upstream *SacI* and a downstream *BglII* site (*BglII* and *BamHI* create compatible overhangs) were added to the FS-HBP2 and MS-HBP1 coding regions by PCR. The PCR 10 consisted of 20 cycles with a 30-second melting step at 95°C, a 30-second primer-annealing step at 50°C and a 30-second extension step at 72°C. The forward primer, in the case of FS-HBP2 was: 5'-AAGGAGCTCAGCATGAAGCTTCTCAT; the reverse primer was: 5'- TATAGATCTCTAGGCAAGCACTTGTG.

15

In the case of MS-HBP1 the forward primer was:
5'-GCAGGAGCTCGGCACGAG, and the reverse primer was:
5'-TATAGATCTGGTTCTGAGCTGGTGCTG.

20 Following PCR, the derived cDNAs were inserted into the vector. A Gln-Ile-(HIS)₆ sequence was thus added to the carboxyterminus of the MS-HBP1 translation product, and Ile-(His)₆ to the FS-HBP2 translation product.

25 The baculovirus expression system was used for expression of the three tagged polypeptides. *Spodoptera* (Sf21) cells were transfected with the transfer vectors and baculovirus (BacPak6; Clontech). Recombinant virus was amplified as according to Kitts and Possee (1993). The VABPs are clearly 30 secretion products since they are mainly found in the culture medium of transfected cells as well as in saliva.

The coding region of (mature) FS-HBP2 was also cloned into the pET-23a(+) expression vector. The sequences from 35 position (a) to (b) and from (c) to (d) in Figure 7 were deleted in truncated versions of bacterially-expressed FS-HBP2. The N-terminally truncated protein was obtained by PCR on the pACC129.1-HIS plasmid containing FS-HBP2, using the

forward primer 5'-TATGGATCCTTCACTGCGTGGGTGTT and the reverse primer 5'- TATAGCGGCCGCCGGCTAGTGATGGTGATGATGAT. The PCR product was cut with *Bam*HI and *Not*I and inserted in between the *Bam*HI and *Not*I sites of the pET-23a(+) vector.

5

In the case of the carboxyterminal truncation, the complete FS-HBP2 coding region was inserted into the pET23a(+) vector, using the forward primer:

10 5'- TATAGGATCCGGGAGCTCCAATCAGCCAGATTGGGC and the reverse primer:

15 5'- TATAGCGGCCGCCGGCTAGTGATGGTGATGATGAT. The PCR product was cut with *Bam*HI and *Not*I and inserted between the *Bam*HI and *Not*I sites of the pET-23a(+) vector. The plasmid (with insert) was then used as a template for PCR with the inverse primers; 5'-TATATGGTACCCATCATCATCACACCACATCAC and 5'-ATATATGGTACCGTTGTCGTAATCCGTAGTC. This resulted in amplification of the complete plasmid minus the region to be deleted. Religation was performed after cutting with *Kpn*I (the primers contain *Kpn*I sites). The original, unamplified 20 plasmid was destroyed by digestion with *Dpn*I, prior to transformation. All PCRs consisted of 20 cycles with a 30-second melting step at 95°C, a 30-second primer-annealing step at 50°C and a 90 second extension step at 72°C.

25 2) Protein purification and production of antisera

60 hours after infection of the Sf21 cells, the culture medium was collected, cells and cellular debris were spun down (2,000g, 10 minutes) and the supernatant was fractionated by (NH₄)₂SO₄ precipitation. rFS-HBP1 and rFS-HBP2 precipitated in the 50 to 80% (NH₄)₂SO₄ fraction and MS-HBP1-His in the 65-100% fraction. The pellets were washed in 100% (NH₄)₂SO₄, redissolved in PBS and purified over Ni-agarose columns (Qiagen) according to Janknecht et al., (1991). The histidine-tagged proteins were eluted 35 using imidazole. Centricon 10 concentrators (Amicon) were used to concentrate the eluans and for buffer exchange. The purified protein was stored at -20°C in PBS.

For production of polyclonal antisera, purified recombinant protein (ca. 2 μ g in 150 μ l PBS) was mixed with an equal volume of Montanide ISA 50 adjuvant (Seppic, France) and subcutaneously injected into Dunkin Hartley guinea pigs.

- 5 This procedure was repeated every 10 days. Serum was collected 10 days after the 4th injection.

3) Electrophoresis and Western Blotting

Salivary glands (and other tissues) were excised from ticks 10 at different time points of the feeding period, and homogenised in PBS. The homogenates were centrifuged at 10,000g for 5 minutes and the supernatants were submitted to sodium dodecyl sulphate-polyacrylamide electrophoresis (SDS-PAGE; Laemmli, 1970).

15 Figure 7 shows a 12% SDS-PAGE gel over which rFS-HBP1, rFS-HBP2 and rMS-HBP1 were run. rFS-HBP1 and rFS-HBP2 run on agarose with apparent molecular masses of ~21 and ~24kDa respectively, whilst rMS-HBP2 runs at ~22kDa.

20 For Western blotting, proteins were transferred to nitrocellulose (Gelman Sciences) by means of semi-dry electroblotting (Kyhse-Anderson, 1984) using an AE-6675 Horizblot apparatus (Atto Corporation, Japan). FS-HBP1, FS-25 HBP2 and MS-HBP1 were identified using the antisera produced in guinea pigs (see above), in combination with goat anti-guinea pig immunoglobulins conjugated to alkaline phosphatase (Sigma). Kinase activity was visualised with nitro blue tetrazolium salt and 5-bromo-4-chloro-3-indolyl phosphate (Blake et al., 1984).

35 Eventual asparagine-linked glycosylation of proteins was studied by means of mobility shift assays. SDS-PAGE and immunoblotting were carried out with salivary gland extracts and recombinant protein samples, before and after treatment with N-glycosidase F (PNGase F; New England Biolabs), an endoglycosidase that hydrolyses all common types of Asn-glycan chains from glycoproteins (Maley et al., 1989). Only

MS-HBP1 shows any downward shift in mobility in SDS-PAGE gels upon treatment with N-glycosidase F, indicating that it is a glycoprotein. The downward shift corresponds to a 2-3kDa change in molecular weight.

5

Figure 8 shows western blots containing salivary gland extracts of female (A and B) and male (C) ticks taken at different time points of the adult feeding period and resolved over a 12% SDS-PAGE gel. Anti-FS-HBP1 (A) and 10 anti-FS-HBP2 (B) sera show positive reactions from the first to the third day after attachment (p.a.). The anti-MS-HBP1 serum (C) detected MS-HBP1 from the first day p.a. until the end of the feeding period.

15 4) N-terminal sequencing

The amino terminal sequences of purified rFS-HBP1, rFS-HBP2 and rMS-HBP1 were determined at the MRC Immunochemistry Unit of the Department of Biochemistry of the University of Oxford. Samples were run on SDS-PAGE gels according to the 20 method of Schägger and von Jagow (1987) and electroblotted onto ProBlott membranes (Applied Biosystems, Warrington, England). The membranes were stained with Coomassie brilliant blue and the bands of interest were excised and sequenced, according to Matsudaira (1987). Electroblotted 25 samples were run on an Applied Biosystems 494A "Procise" protein sequencer (Perkin-Elmer, Applied Biosystems Division, Warrington, UK) using an Applied Biosystems "Mini-Blott" cartridge (onto which the membrane pieces were inserted). The manufacturer's recommended programme for 30 membrane-bound samples was used for sequencing.

Example 4: Characterisation of proteins

1) Histamine binding assays

35 The purified recombinant proteins were submitted to histamine binding assays as set out in Warlow and Bernard (1987). This method uses protein precipitation to separate free from bound ligand (radiolabelled histamine) by addition

of polyethylene glycol (M_w 8000) and centrifugation. In all experiments, thin-layer chromatographs were run in an acetate-ammonia solvent system after a four hour incubation period to ensure that no metabolism of histamine had 5 occurred.

Saturable binding of ^3H -histamine was obtained with all 3 rVABPs (Figures 9Ai, 9Bi and 9Ci). Scatchard plots (Figures 9Aii, 9Bii and 9Cii) show high affinities for rMS-HBP 10 ($K_d=1.2 \times 10^{-9}\text{M}$; SD=0.4; 3 measurements) and for rFS-HBP2 ($K_d=1.7 \times 10^{-9}\text{M}$; SD=0.9), but a lower affinity for rFS-HBP1 ($K_d=7.8 \times 10^{-8}\text{M}$; SD=1.5), suggesting that binding histamine may not be the primary function of this protein.

- 15 There is some evidence for co-operative binding in the case of rMS-HBP1. When samples containing ^3H -histamine (~0.3pmol; 11,200cpm) and excess amounts of rMS-HBP1 (~100pmol) were supplemented with small amounts of histamine (0.5pmol), a significant increase of bound radioligand was measured 20 (7,560 \pm 110cpm, compared to 6,840 \pm 150cpm; 5 measurements), indicating an enhanced binding capacity. Co-operative binding is in agreement with the dimer or polymer nature of MS-HBP1. Indeed, MS-HBP1 appears to form intermolecular disulphide bridges; it has a lower mobility 25 on SDS gels when reducing agent is left out of the loading buffer. The FS-HBPs seem to have only intramolecular disulphide bonds, as is suggested by the higher mobilities in the absence of reducing agent.
- 30 In a competition experiment (carried out in triplicate), a series of histamine-like compounds [histamine, imidazole, serotonin, dopamine, the H1-receptor agonist betahistine, the H1 antagonists chlorpheniramine and pyrilamine, the H2-agonist dimaprit, and the H2 antagonists ranitidine and 35 cimetidine] were added to each of the rVABPs in 1000-fold the amounts at which cold histamine displaces more than 95% of ^3H -histamine from the binding sites. The histamine-like compounds caused little or no displacement of radioligand,

indicating that the VABPs bind histamine specifically and in a different manner from the H1 and H2 receptors.

FS-HBP2 was expressed in the pET-23a(+) vector in
5 AD494(DE3)pLySS bacteria (Novagen). Bacterially-expressed
FS-HBP2 binds histamine with a somewhat lower affinity
($K_d=0.6-0.9 \times 10^{-8} M$) than that expressed in the baculoviral
system. Truncated versions of the protein (see above) that
lack either the 45 N-terminal amino acids or the 28 C-
10 terminal amino acids do not bind to histamine at all. This
suggests that the overall structure of FS-HBP2 is important
for histamine binding and that the binding site is more
likely to be determined by dispersed residues, rather than
a stretch of consecutive amino acids located somewhere on
15 an α -helix or β -sheet.

2) Contraction-inhibition

Contraction-inhibition experiments (Figure 10) were carried
out on guinea pig ileum suspended in a 10ml chamber
20 containing aerated Krebs solution. Contractions (recorded
as peaks) were induced by adding 1.25nmol histamine (H) to
the chamber. After a peak was reached histamine was washed
away with Krebs solution (W), allowing the ileum to relax.
Contraction was substantially reduced by adding - 2nmol rFS-
25 HBP2 (F2) together with the histamine. - 2nmol of rFS-HBP1
had no significant effect (data not shown). - 4nmol
(monomer amount) of rMS-HBP1 (M) added together with
histamine completely inhibited contraction, even after extra
histamine (xH) was added.

30

The rMS-HBP1 and rFS-HBP2 proteins are strong enough binders
to compete with histamine with the H1 receptors of guinea
pig ileum (see Figure 10). In accordance with its
relatively low affinity, little or no inhibition of ileum
35 contraction was observed with rFS-HBP1.

REFERENCES

27

Blake, M.S., Johnston, K.H.; Russell-Jones, G.J., Gotschlich, E.C. (1984) A rapid, sensitive method for detection of alkaline-phosphatase-conjugated anti-antibody on Western blots. *Anal. Biochem.* 136, 175-179.

Falus A. (1994) Histamine and Inflammation. R.G. Landes Company, Austin, pp.139.

Goode, B.L. and Feinstein, S.C. (1992) "Speedprep" purification of template for double-stranded DNA sequencing. *BioTechniques* 12, 374-375.

Janknecht, R., de Martynoff, G., Lou, L., Hipskind, R.A., Nordheim, A. and Stunnenberg, H.G. (1991) Rapid and efficient purification of native histidine-tagged protein expressed by recombinant vaccinia virus. *PNAS* 88, 8972-8976.

Jones, L.D., Davies, C.R., Steele, G.M. and Nuttall, P.A. (1988). The rearing and maintenance of ixodid and argasid ticks in the laboratory. *Animal Technology* 39, 99-106.

Kitts, P.A. and Possee, R.D. (1993) A method for producing recombinant baculovirus expression vectors at high frequency. *BioTechniques* 14, 810-817.

Kyhse-Anderson, J. (1984) Electroblotting of multiple gels: A simple apparatus without buffer tank for rapid transfer of proteins from polyacrylamide to nitrocellulose. *J. Biochem. Biophys. Methods* 10, 203-209.

Laemmli, V.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 277, 680-685.

Livingstone, C. and Jones, I (1989) Baculovirus expression vectors with single strand capability. Nucleic Acids Res. 17, p. 2366.

Maley, F., Trimble, R.B., Tarentino, A.L. and Plummer, T.H, Jr. (1989) Characterization of glycoproteins and their associated oligosaccharides through the use of endoglycosidases. Anal. Biochem. 180, 195-204.

Matsudaira, P. (1987). Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. J. Biol. Chem. 262, 10035-10038.

Mierendorf, R.C. and Pfeffer, D. (1987) Direct sequencing of denatured plasmid DNA. Methods Enzymol. 152, 556-562.

Program Manual for the Wisconsin Package, version 8 (1994) Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA 53711.

Protein Expression in Current Protocols in Molecular Biology (Chapter 16). Editors : F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, K. Struhl. Published by John Wiley and Sons, Inc.

Ribeiro, J.M. and Walker, F.A. (1994) High-affinity histamine-binding and antihistaminic activity of the salivary nitric oxide-carrying heme protein (nitrophorin) of Rhodnius prolixus. J. Exp. Med. 180, 2251-2257.

Sambrook J., Fritsch E.F. and Maniatis T. (1989) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory.

Sanger F. and Coulson A.R. (1975). A rapid method for determining sequences in DNA by primed synthesis with DNA polymerase. *J. Mol. Biol.* 94, 441-448.

Schägger, H. and von Jagow, G. (1987) Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100kDa. *Anal. Biochem.* 166, 368-379.

Short J. M., Fernandez J.M., Sorge J.A. and Huse W.D. (1988) λ ZAP: a bacteriophage λ expression vector with in vivo excision properties. *Nucl. acids Res.* 16, 7583-7600.

Towbin, H., Staeholin, T. and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets : Procedure and some applications. *Proc. Natl. Acad. Sci. USA* 76: 4350-4354.

Wang, H. and Nuttall, P.A. (1995). Immunoglobulin G binding proteins in male *Rhipicephalus appendiculatus* ticks. *Parasite Immunology* 17: 517-524.

Warlow, R.S. and Bernard, C.C.A. (1987). Solubilization and characterization of moderate and high affinity histamine binding sites on human blood mononuclear cells. *Molec. Immun.* 24, 27-37.

CLAIMS

1. A vasoactive amine binding protein (VABP) that specifically binds to vasoactive amines with a dissociation constant of less than 10^{-7} M and which belongs to the same protein family as MS-HBP1, FS-HBP1, FS-HBP2 and D.RET6.
2. A VABP according to claim 1, that is derived from blood-feeding ectoparasites, spiders, scorpions, snakes or venomous animals.
3. A VABP according to claim 1, that is derived from ticks.
4. A VABP according to claim 1, that is derived from the ticks *Rhipicephalus appendiculatus* or *Dermacentor reticularis*.
5. A VABP according to any one of the preceding claims that specifically binds to histamine.
6. Any one of the VABPs FS-HBP1, FS-HBP2, MS-HBP1 and D.RET6.
7. A functionally-equivalent derivative or fragment of any one of the VABPs of any one of claims 1 to 6.
8. A VABP, derivative or fragment (VABM) according to any preceding claim that is expressed in recombinant form.
9. A VABM according to claim 7 or claim 8 wherein the VABP has been genetically or chemically fused to one or more peptides or polypeptides.
10. A VABM according to any one of claims 1 to 9 that is bound to a support, such as a resin.
11. A nucleic acid molecule which encodes a VABM according

to any one of claims 1 to 9 or which hybridises with such a VABM-encoding molecule.

12. The nucleic acid of claim 11 which comprises DNA, cDNA
5 or RNA.

13. The nucleic acid of claim 11 or claim 12 which
comprises DNA.

10 14. A cloning or expression vector comprising a nucleic
acid molecule according to any one of claims 11 to 13.

15. The vector of claim 14 which is virus based.

15 16. The vector of claim 15 which is baculovirus based.

17. A host cell transformed or transfected with the vector
of any one of claims 14 to 16.

20 18. A transgenic animal that has been transformed by a
nucleic acid molecule according to any one of claims 11 to
13.

25 19. A method of preparing a protein according to any one of
claims 1 to 9, comprising expressing a vector according to
any one of claims 14 to 16 in a host cell and culturing said
host cells under conditions where said protein is expressed,
and recovering said protein thus produced.

30 20. A kit suitable for the quantification of histamine
levels in a body fluid of a human or animal comprising a
VABM according to any one of claims 1 to 10 and detection
means.

35 21. A VABM according to any one of claims 1 to 10 for use
in therapy.

22. A VABM according to any one of claims 1 to 10 for use

in binding vasoactive amines in humans or animals.

23. A VABM according to any one of claims 1 to 10 for use in binding histamine in humans or animals.

5

24. The use of a VABM according to any one of claims 1 to 10 for the detection of vasoactive amines in humans or animals.

10 25. The use of a VABM according to any one of claims 1 to 10 for the detection of histamine in humans or animals.

26. The use of a VABM according to any one of claims 1 to 10 in a vaccine.

15

27. A VABM according to any one of claims 1 to 10 for use as an anti-histamine agent.

20 28. A VABM according to any one of claims 1 to 10 for use as an anti-inflammatory drug.

25 29. The use of a VABM according to any one of claims 1 to 10 in conjunction with a pharmaceutically-acceptable carrier in the manufacture of a medicament for the treatment of inflammation in humans or animals.

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FIG. 1

FS-HBP1

T3→

1 AGAAAGCCAACATGAAGCTTCTGCTCTCTTGCCTTCGTCTTAGCTCTCAGCCAAGTTA 60
 M K L L S L A F V L A L S Q V K

61 AAGCCGATAAGCCAGTTGGCGGATGAAGCGCAAACGGGAACACCAAGACGCCCTGGA 120
 A D K P V W A D E A A N G E H Q D A W K
 ↑

121 AGCATCTCCAAAAACTCGTTGAAGAGAATTACGACTTGATAAAAGCCACCTACAAGAACG 180
 H L Q K L V E E N Y D L I K A T Y K N D

181 ACCCAGTTGGGTAAACGACTTCACTTGCGTGGGTACTGCAGCGCAGAATTGAAACGAGG 240
 P V W G N D F T C V G T A A Q N L N E D

241 ACGAGAAGAACGTTGAAGCATGGTTATGTTATGAATAATGCTGATACCGTATACCAAC 300
 E K N V E A W F M F M N N A D T V Y Q H

301 ATACTTTGAAAAGGCAGCTCCTGATAAAATGTACGGTTACAATAAGGAAACGCCATCA 360
 T F E K A T P D K M Y G Y N K E N A I T

361 CATATCAAACAGAGGATGGCAAGTTCTCACAGACGTCCTTGCAATTCTGACGACAATT 420
 Y Q T E D G Q V L T D V L A F S D D N C

421 GCTATGTCATCTACGCTCTGGCCCAGATGGAAGTGGAGCAGGTTACGAACTCTGGCTA 480
 Y V I Y A L G P D G S G A G Y E L W A T

481 CCGATTACACGGATGTTCCAGCCAGTTGTCTAGAGAAGTTCAATGAGTATGCTGCAGGTC 540
 D Y T D V P A S C L E K F N E Y A A G L

541 TGCCGGTACGGGACGTATACACAAGTGATTGCCCTCCAGAATAACTTGGCATATCGTAA 600
 P V R D V Y T S D C L P E *

601 TTTCAACTCAAAGTGTGTTATTGTCAGCATATGTCAGTGAGTGTGATGAGTGCCTTC 660

661 GATGATGCCATTCACTAGGTTGGGTGTTGGTACTTATGGTCACTGCCGACGGCCA 720

721 GCACGAGTACTGAAAATAAAAGTATTCTGAAATCGGAAAAA 770 ←T7

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FIG. 2

FS-HBP2

T3→	1	GCCGCGACGGAACCTCGAAGGAAGTCAGCATGAAAGCTTCTCATACTCTCTTGGCCCTCG M K L L I L S L A L V	60
61	TCCTCGCCCTCAGCCAGGTTAAGGGAAATCAGCCAGATTGGGCCATGAAGCGGCAAATG L A L S Q V K G N Q P D W A D E A A N G	120	
121	GTGCACACCAAGACGCCCTGGAAGAGTCAGTAAAGCGGACGTTGAAAACGTTACTACATGG A H Q D A W K S L K A D V E N V Y Y M V	180	
181	TGAAGGCCACCTATAAGAACATGACCCAGTGTTGGGCAATGACTTCACTTGCGTGGGTGTTA K A T Y K N D P V W G N D F T C V G V M	240	
241	TGGCAAATGATGTCAACGAGGGATGAGAAGAGCATTCAAGCAGAGTTTGTGTTATGAATA A N D V N E D E K S I Q A E F L F M N N	300	
301	ATGCTGACACAAACATGCAATTGCCACTGAAAAGGTGACTGCTGTTAAATGTATGGTT A D T N M Q F A T E K V T A V K M Y G Y	360	
361	ACAATAGGGAAAACGCCCTCAGATACGAGACGGAGGATGCCAAGTTTACAGACGTCA N R E N A F R Y E T E D G Q V F T D V I	420	
421	TTGCATACTCTGATGACA <u>ACTGCGATGT</u> CAT <u>CTACGTTCTGGCACAGACGGAAATGAGG</u> A Y S D D N C D V I Y V P G T D G N E E	480	
481	AAGGTTACGA <u>ACTATGGACTACGGATTACGACAACATTCCAGCCAATGTTAAATAAGT</u> G Y E L W T T D Y D N I P A N C L N K F	540	
541	TTAATGAGTAGCGCTGTAGGTAGGGAGACAAGGGATGTATT <u>CACAAGTGCTTGCCTAGAGT</u> N E Y A V G R E T R D V F T S A C L E	600	
601	<u>AATAACTTCAGAATGTCGTTCTTC</u> AAAGCGAAAA <u>ACCAACAAATGTAAACATCGGCTTGC</u> → ←	660	
661	TGTGCTCGACGTAGCCAGCGATAATGTTGTTCTGGGTTCTGGGTTGGATA <u>ACTTTT</u>	720	
721	AGCCACTGCCGAAGAGCTGTAAAGGTAA <u>ATGAAAAAATGTTCAAGAGTGTGAAAAAA</u>	780	
781	AAAAAAAAAAAAAAA 793 ←T7		

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FIG. 3

MS-HBPI

T3→		
1	AAAGCACTCAACATGAAGGTTCTTGTGGTCTGGAGCTGCTCTGCCAGAACATGCA M K V L L L V L G A A L C Q N A	60
61	GATGCAAACCCAAACATGGCGAACGAAGCTAAATTGGGATCCTACCAAGACGCCCTGGAAG D A N P T W A N E A K L G S Y Q D A W K ↑	120
121	AGCCTTCAGCAAGACCAAAACAAGAGATACTATTTGGCACAAGCGACACAAACGACTGAC S L Q Q D Q N K R Y Y L A Q A T Q T T D	180
181	GGCGTATGGGTGAAGAGTTACTTGTGTGAGTGTACGGCTGAGAAGATTGAAAGAAA G V W G E E F T C V S V T A E K I G K K	240
241	AAACTTAACGCTACGATCCTCTATAAAAATAAGCACCTTACTGACCTGAAAGAGAGTCAT K L N A T I L Y K N K H L T D L K E S H _____	300
301	GAACAATCACTGTCTGGAAAGCATACGACTACACAAACGGAGAATGGCATCAAGTACGAG E T I T V W K A Y D Y T T E N G I K Y E	360
361	ACGCAAGGGACAAGGACCGAGACTTTCGAAGATGTCTTGATTCTCTGATTACAAGAAC T Q G T R T Q T F E D V F V F S D Y K N	420
421	TGCATGTAATTCGTTCCCAGAGAGAGAGAGAGAGCGACGAGGGCGACTATGAATTGTGG C D V I F V P K E R G S D E G D Y E L W	480
481	GTTAGTGAAGACAGGATTGACAAGATTCCGATTGCTGCAAGTTACGATGGCGTACTTT V S E D K I D K I P D C C K F T M A Y F	540
541	GCCAAACAGCAGGAGAACGCGGTTGTAATGTATACACTGACTCATCATGCAAACAGCA A Q Q Q E K T V R N V Y T D S S C K P A	600
601	CCAGCTCAGAACTGATATTCTGGTAATGCTGAAACCGTAATGGTCGACCTGCAGTCTAG P A Q N *	660
661	AAACATTTACCAACCACACGGTAGTTACCGTAGTTCTTAGGTCTGTTCTTGA	720
721	ATAAAAATAGTCCCTGCATTGACAAAAAAAAAA	753

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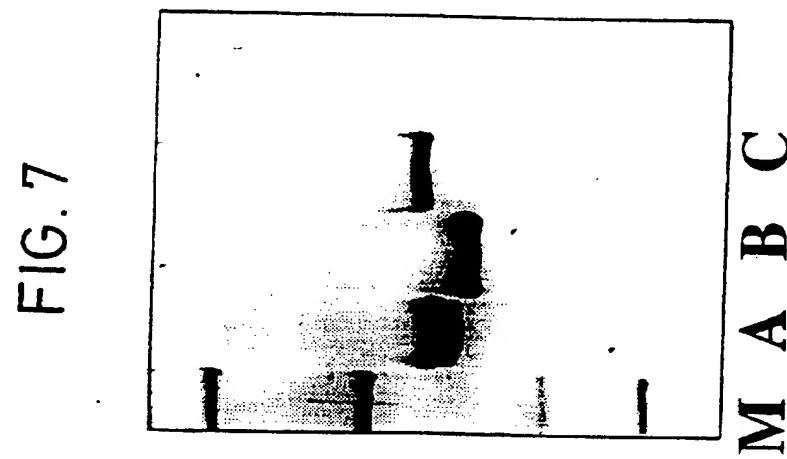
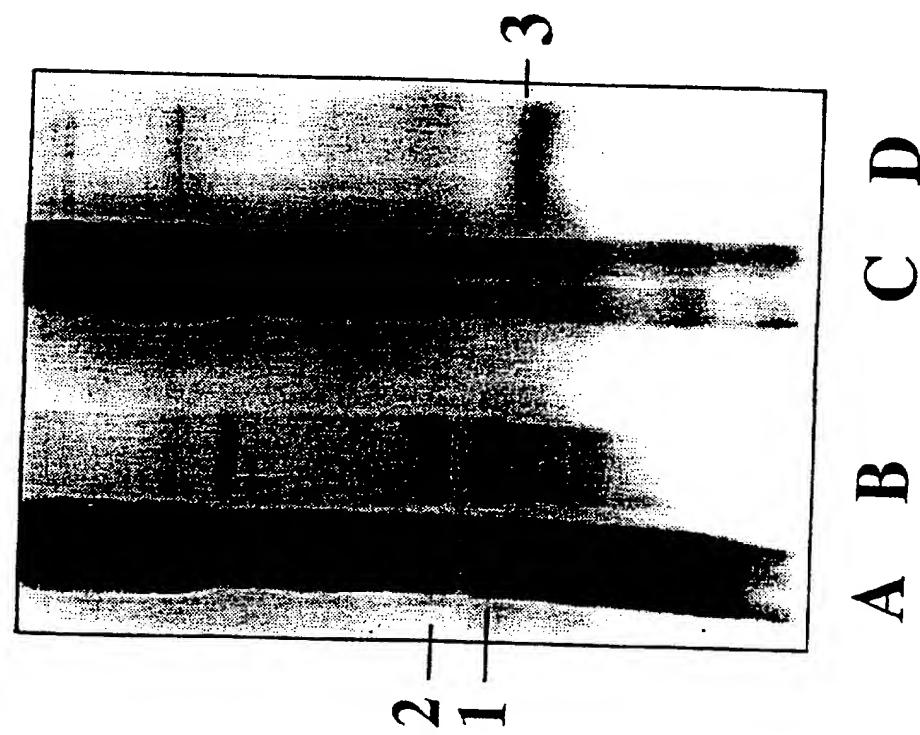
FIG. 4

T3→

1	ATGAAGATGCAGGTAGTGCTCTTACCTTACCTTGTAGCGCCGCCCTGCCACTCAAGCG	60
1	M K M Q V V L L T F V S A A L A T Q A	20
61	GAGACTACATCTGC 21 E T T S A K A G E N P L W A H E E L L G	120
	↑	40
121	AAATATCAAGATGC 41 K Y Q D A W K S I D Q G V S V T Y V L A	180
		60
181	AAGACAACATATGAGAATGACACAGGATCATGGGATCCC 61 K T T Y E N D T G S W G S Q F K C L Q V	240
	→ ←	80
241	CAAGAAATAGAAAAGGAAGAAGACTATA 81 Q E I E R K E E D Y T V T S V F T F R N	300
		100
301	GCGTCTTC 101 A S S P I K Y Y N V T E T V K A V F Q Y	360
		120
361	GGATACAAAAACATAAGGAATGCAATTGAATACCA 121 G Y K N I R N A I E Y Q V G G G L N I T	420
		140
421	GACACGCTCATTTCACTGATGGAGAATTATGC 141 D T L I F T D G E L C D V F Y V P N A D	480
	→ ←	160
481	CAAGGTTGTGAGCTCTGGGTCAAAAAGAGTC 161 Q G C E L W V K K S H Y K H V P D Y C T	540
		180
541	TTCGTGTCAATGTTTCTGTGC 181 F V F N V F C A K D R K T Y D I F N E E	600
		200
601	TGTGTTATAACGGCGAACCTGGTTAAAGC 201 C V Y N G E P W L *	660
		220
661	TAGTAAGTACTAATAGCAAGTAGTTGAATA ←T7	719

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FIG. 5



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FIG. 6

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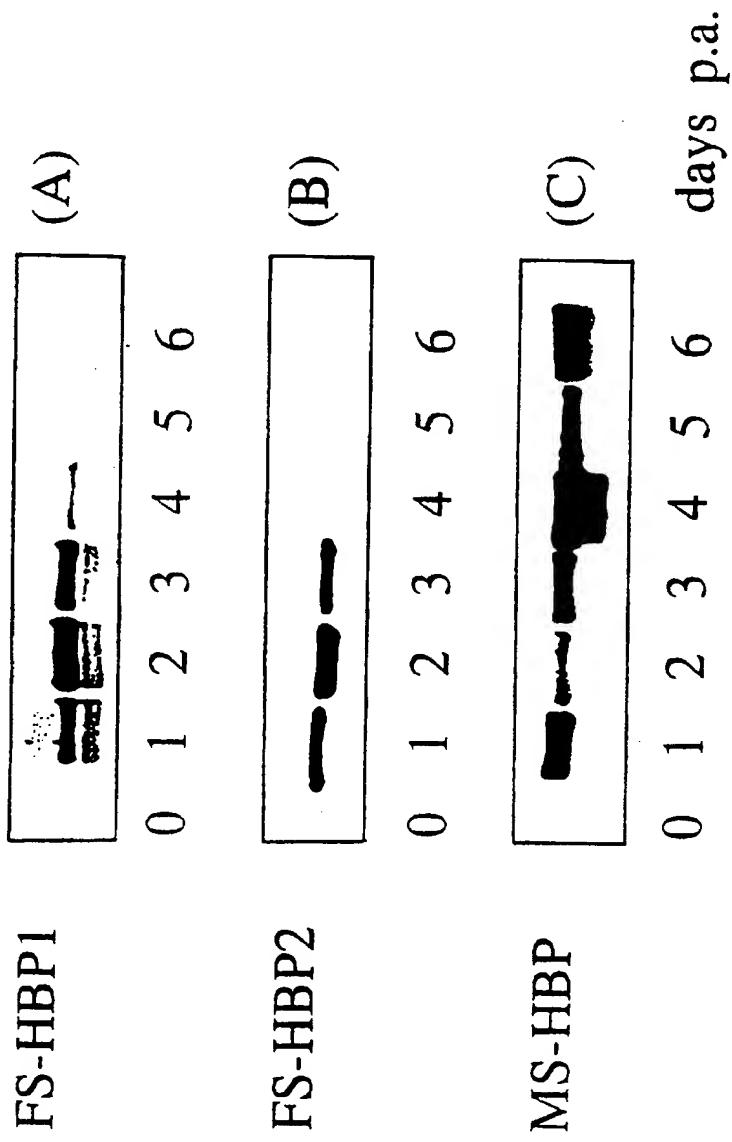
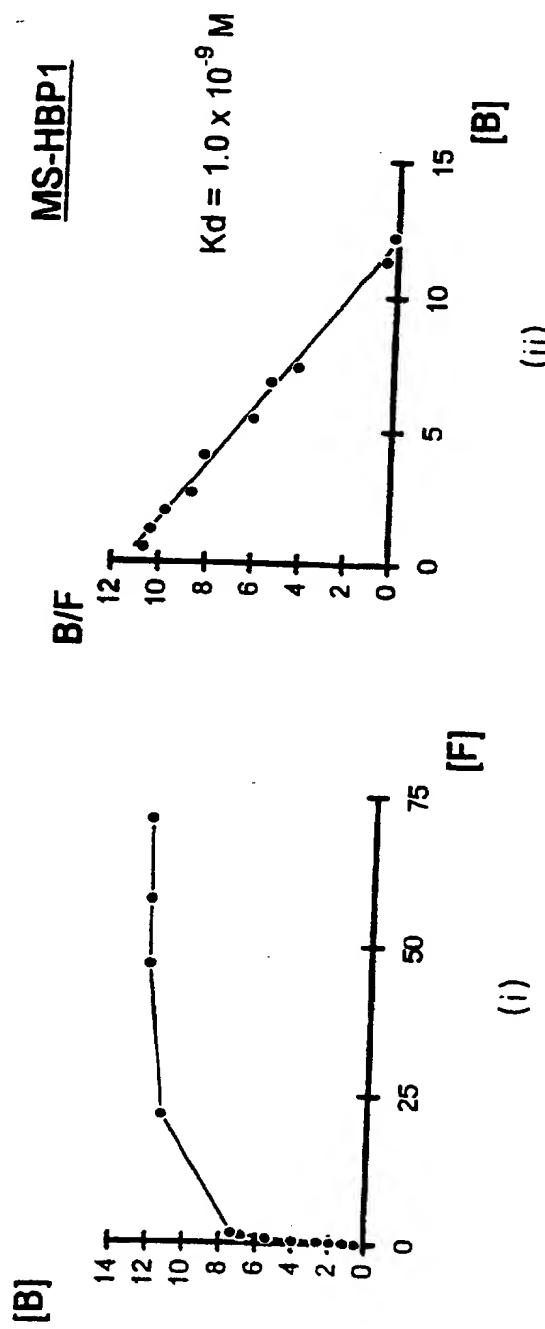


FIG. 8

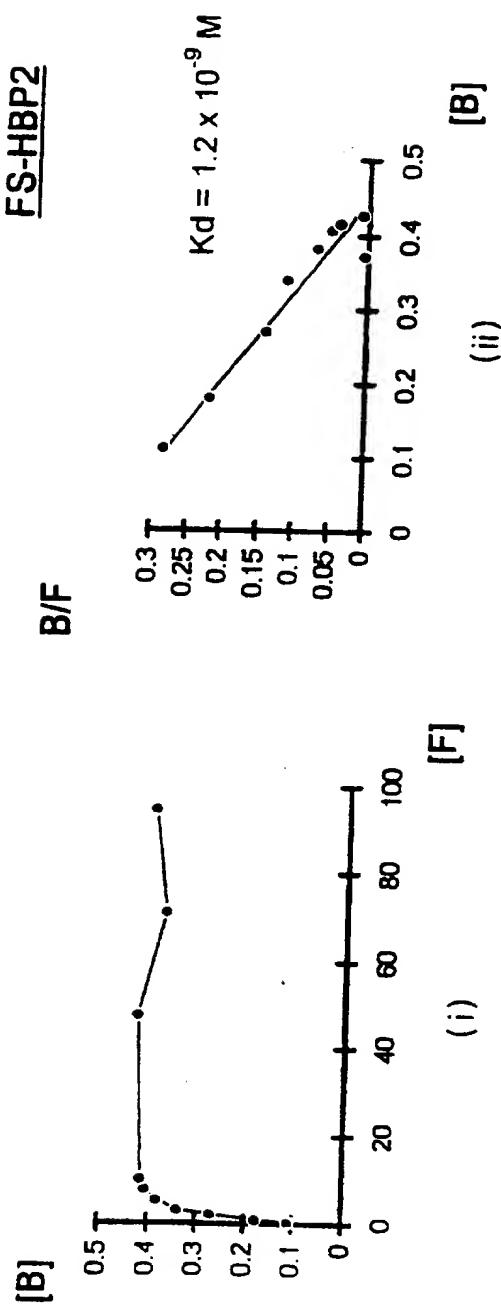
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FIG. 9A



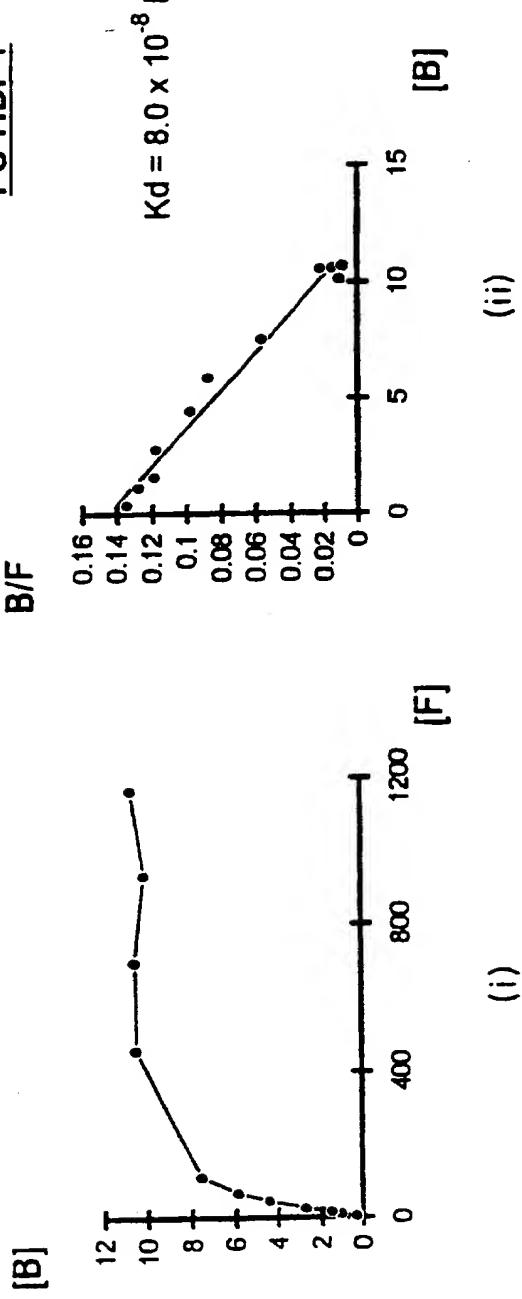
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FIG. 9B



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FIG. 9C
FS-HBP1



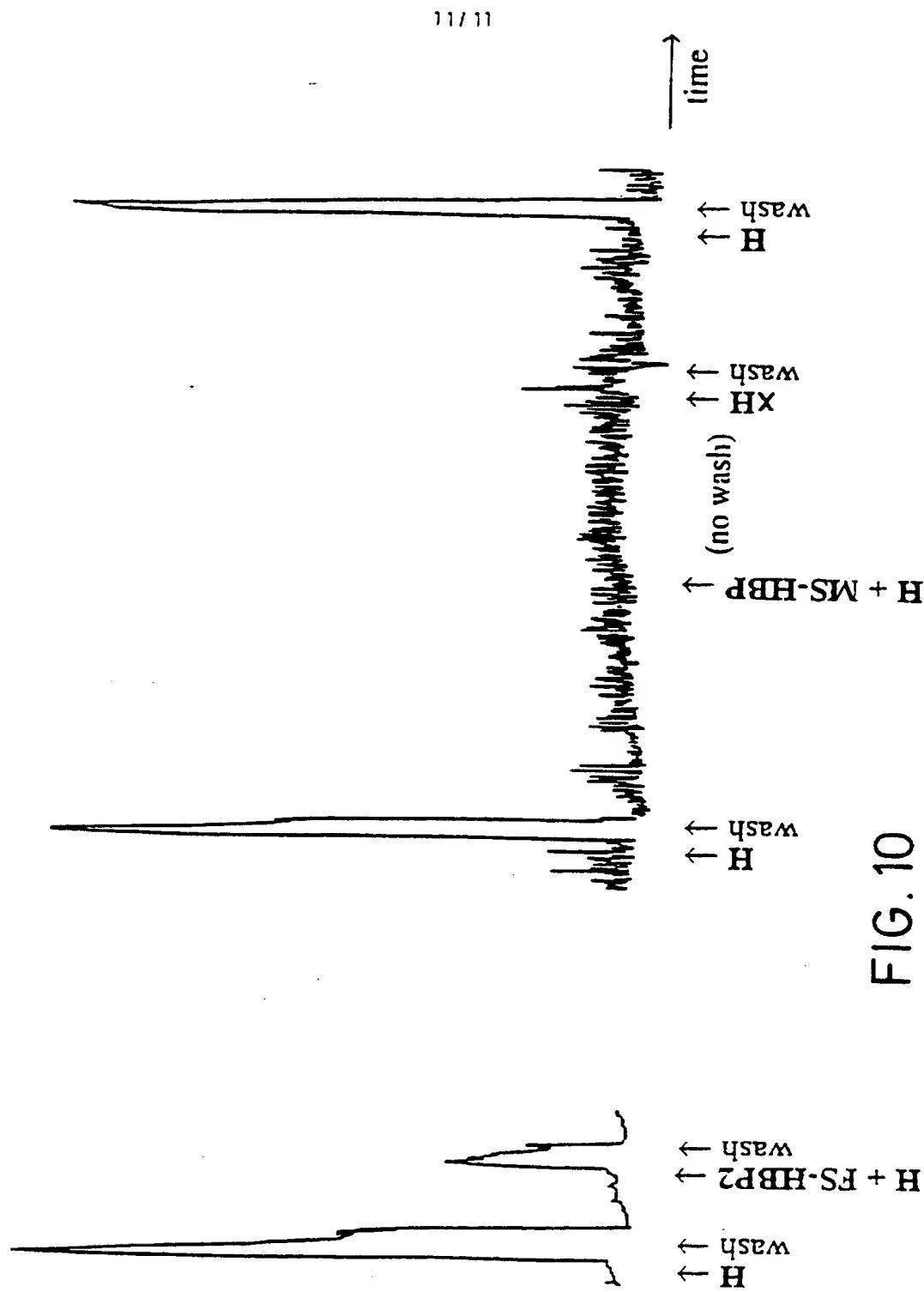


FIG. 10

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(51) International Patent Classification ⁶ : C12N 15/12, 15/62, 15/86, 5/10, C07K 14/435 A01K 67/027, 38/17, G01N 33/68		A3	(11) International Publication Number: WO 97/44451 (43) International Publication Date: 27 November 1997 (27.11.97)
<p>(21) International Application Number: PCT/GB97/01372</p> <p>(22) International Filing Date: 19 May 1997 (19.05.97)</p> <p>(30) Priority Data: 9610484.9 18 May 1996 (18.05.96) GB 9707844.8 18 April 1997 (18.04.97) GB </p> <p>(71) Applicant (for all designated States except US): OXFORD VACS LTD. [GB/GB]; 4/5 Grosvenor Place, Hyde Park Corner, London SW1X 7DL (GB).</p> <p>(72) Inventors; and</p> <p>(75) Inventors/Applicants (for US only): PAESEN, Guido, Christian [BE/GB]; 12 Mansfield Road, Oxford OX1 3TA (GB). NUTTALL, Patricia, Ann [GB/GB]; 1 Manor Farm, Culham, Oxon OX14 4NP (GB).</p> <p>(74) Agent: MERCER, Christopher, Paul; Carpmaels & Ransford, 43 Bloomsbury Square, London WC1A 2RA (GB).</p>		<p>(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU. ARIPO patent (GH, KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).</p> <p>Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p> <p>(88) Date of publication of the international search report: 19 February 1998 (19.02.98)</p>	
<p>(54) Title: VASOACTIVE AMINE BINDING MOLECULES</p> <p>(57) Abstract</p> <p>According to the present invention, there are provided vasoactive amine binding proteins (VABPs) that specifically bind to vasoactive amines with a dissociation constant of less than 10^{-7} M and which belong to the same protein family as MS-HBP1, FS-HBP1, FS-HBP2 and D.RET6.</p>			

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INTERNATIONAL SEARCH REPORT

Int. Appl. No.
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A. CLASSIFICATION OF SUBJECT MATTER					
IPC 6 C12N15/12 C12N15/62 C12N15/86 C12N5/10 C07K14/435 A01K67/027 A61K38/17 G01N33/68					
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X	GB 2 283 239 A (UCB SA) 3 May 1995 see the whole document ---				1,5-9, 11-17, 19-29
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A	<p>WANG H. AND NUTTALL P.: "Comparison of the proteins in salivary glands, saliva and haemolymph of <i>Rhipicephalus appendiculatus</i> female ticks during feeding" PARASITOLOGY, vol. 109, no. pt.4, November 1994, pages 517-523, XP002050816 see the whole document</p> <p>-----</p>	1-29
A	<p>WO 96 11271 A (HESKA CORP ;FRANK GLENN R (US); HUNTER SHIRLEY WU (US); WALLENFELS) 18 April 1996 see the whole document</p> <p>-----</p>	1-29

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GB 2283239 A	03-05-95	NONE		
WO 9611271 A	18-04-96	US 5646115 A		08-07-97
		AU 3895195 A		02-05-96
		CA 2201482 A		18-04-96
		EP 0784682 A		23-07-97
		ZA 9508469 A		13-05-96